JOURNAL OF BACTERIOLOGY, June 1997, p. 3892–3898 Vol. 179, No. 12
0021-9193/97/$04.00+0

Evidence that the N-Terminal Part of the S-Layer Protein from *Bacillus stearothermophilus* PV72/p2 Recognizes a Secondary Cell Wall Polymer

WOLFGANG RIES, CHRISTOPH HOTZY, INGRID SCHOCHER, UWE B. SLEYTR, AND MARGIT SÁRA*

Zentrum für Ultrastrukturforschung und Ludwig Boltzmann-Institut für Molekulare Nanotechnologie, Universität für Bodenkultur, Vienna, Austria

Received 21 January 1997/Accepted 1 April 1997

The S-layer of *Bacillus stearothermophilus* PV72/p2 shows oblique lattice symmetry and is composed of identical protein subunits with a molecular weight of 97,000. The isolated S-layer subunits could bind and recrystallize into the oblique lattice on native peptidoglycan-containing sacculi which consist of peptidoglycan of the A1γ chemotype and a secondary cell wall polymer with an estimated molecular weight of 24,000. The secondary cell wall polymer could be completely extracted from peptidoglycan-containing sacculi with 48% HF, indicating the presence of phosphodiester linkages between the polymer chains and the peptidoglycan backbone. The cell wall polymer was composed mainly of GlcNAc and ManNAc in a molar ratio of 4:1, constituted about 20% of the peptidoglycan-containing sacculus dry weight, and was also detected in the fraction of the S-layer self-assembly products. Extraction experiments and recrystallization of the whole S-layer protein and proteolytic cleavage fragments confirmed that the secondary cell wall polymer is responsible for anchoring the S-layer subunits by the N-terminal part to the peptidoglycan-containing sacculi. In addition to this binding function, the cell wall polymer was found to influence the in vitro self-assembly of the guanidinium hydrochloride-extracted S-layer protein. Chemical modification studies further showed that the secondary cell wall polymer does not contribute significant free amino or carboxylate groups to the peptidoglycan-containing sacculi.

Bacillus stearothermophilus PV72 is a strictly aerobic thermophilic endospore-forming organism. The S-layer protein SbsA of the wild-type strain shows a molecular weight of 130,000 and assembles into a hexagonally ordered lattice type (12, 19, 25, 28, 29, 32). Stable synthesis of the S-layer protein from the wild-type strain during continuous cultivation on complex medium could be achieved only when the organism was grown under oxygen and glucose double limitation (25). On synthetic PVIII medium the dissolved oxygen (DO) concentration had to be maintained at 20 to 30% to ensure stable production of the SbsA. At a higher concentration of DO, S-layer protein synthesis of the wild-type strain was synchronously stopped in the whole culture and completely replaced by synthesis of an S-layer protein with a molecular weight of 97,000 which assembled into an oblique lattice type (28). The *sbsB* gene encoding the S-layer protein of the variant (designated PV72/p2) was recently cloned and sequenced (10). Although increased levels of oxygen are responsible for S-layer variation in *B. stearothermophilus* PV72, the exact mechanism leading to change in S-layer protein expression is still under investigation (27). In contrast to the SbsA, the S-layer protein of the variant showed a typical S-layer homologous (SLH) domain in the N-terminal part (10–12). The two S-layer genes have only a low overall similarity (10).

In general, SLH domains have been identified in the N-terminal part in S-layer proteins from various organisms such as *B. sphaericus*, *B. anthracis*, *Thermoanaerobacter kivui*, and *Thermus thermophilus*, the middle wall protein of *B. brevis* 47, and the Ompα of *Thermotoga maritima* (6, 15, 20). With the exception of the Ompα, all S-layer proteins are in direct contact with the peptidoglycan, suggesting that SLH domains could generally be involved in anchoring the S-layer proteins to this cell surface component (15). In addition to S-layer proteins, SLH domains were also identified at the C-terminal end in cell-associated exoenzymes and exoproteins such as the pullulanase, xylanase, and polygalacturonate hydrolase of *Thermoanaerobacterium thermosulfurigenes* EM1 (17, 18) as well as in the OlpA and OlpB of the cellulosome complex of *Clostridium thermocellum* (13).

Chemical analyses of peptidoglycan-containing sacculi from *B. stearothermophilus* PV72/p6 and PV72/p2 strongly indicated that a secondary cell wall polymer of different composition is incorporated into the peptidoglycan of each organism (25, 29). Although the peptidoglycans of the wild-type strain and the PV72/p2 were of the same chemotype and revealed comparable extents of cross-linking, the SbsB recognized only peptidoglycan-containing sacculi from the variant but not those from the wild-type strain. These results led to the conclusion that not the peptidoglycan but the secondary cell wall polymer is responsible for binding of the S-layer subunits to the rigid cell wall layer (25).

In the present study we provide evidence that a secondary cell wall polymer composed mainly of GlcNAc and ManNAc anchors the S-layer protein from the PV72/p2 by its N-terminal part to the peptidoglycan-containing layer.

MATERIALS AND METHODS

Organism, growth conditions, and preparation of cell wall fragments. *B. stearothermophilus* PV72/p2 was grown in continuous culture on complex SVIII medium at 57°C (25) at a dilution rate of 0.12/h in a 10-liter Biostat E bioreactor

* Corresponding author. Mailing address: Zentrum für Ultrastrukturforschung, Universität für Bodenkultur, Gregor-Mendelstr. 33, 1180 Wien, Austria. Phone: 43 1 47 654 2208. Fax: 43 1 478 91 12. E-mail: sara@edv1.boku.ac.at.

(Braun, Melsungen, Germany). The glucose concentration was 3.0 g/liter of SVIII medium. The pH of the culture was kept constant at 7.2 by adding either 1 N sodium hydroxide or 2 M sulfuric acid. The rate of aeration was adjusted to 4.0 liters of air/min, which corresponded to oxygen-limited growth (DO, 0%). Cells were separated from spent medium by continuous centrifugation (Sepatech 17 RS; Heraeus, Hanau, Germany) at 16,000 × *g* at 4°C. The biomass was stored at −20°C. Preparation of cell wall fragments was carried out according to the procedure described in reference 31 except that cells were broken by ultrasonication. After extraction of plasma membrane remnants with Triton X-100 (0.5% in 50 mM Tris-HCl buffer, pH 7.2) for 10 min at 20°C, cell wall fragments were washed at least four times with 50 mM Tris-HCl buffer (pH 7.2) and once with distilled water. Cell wall fragments were stored at −20°C until use. The S-layer protein contents of lyophilized cell wall fragments were determined according to the method in reference 14 using purified S-layer protein for the standard curve.

Production of S-layer self-assembly products and purification of peptidoglycan-containing sacculi. Wet pellets of cell wall fragments were suspended in a 10-fold volume of a guanidinium hydrochloride (GHCl) solution (5 M GHCl in 50 mM Tris-HCl buffer, pH 7.2) and stirred for 20 min at 20°C. After centrifugation at 40,000 × *g* for 20 min at 10°C, the supernatant containing the extracted S-layer protein was carefully removed, centrifuged twice at 40,000 × *g*, and finally dialyzed against distilled water at 4°C overnight. S-layer self-assembly products were sedimented by centrifugation at 20,000 × *g* for 20 min at 4°C, washed at least three times with distilled water, frozen at −20°C, and lyophilized.

Peptidoglycan-containing sacculi obtained after extraction of the S-layer protein were washed once with 5 M GHCl and twice with 50 mM Tris-HCl buffer (pH 7.2). For further purification, 100-mg wet pellets of peptidoglycan-containing sacculi were suspended in 5 ml of a sodium dodecyl sulfate (SDS) solution (1% in distilled water) and incubated for 30 min at 100°C. After cooling down to 20°C, the suspension was centrifuged at 40,000 × *g* for 30 min at 20°C. The pellets were washed at least five times with distilled water, frozen at −20°C, and lyophilized. The purity of the S-layer protein and peptidoglycan-containing sacculi was checked by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), negative staining, and ultrathin sectioning. Electron microscopy was performed as described in a previous paper (19).

Extraction of the secondary cell wall polymer from native peptidoglycan-containing sacculi. Peptidoglycan-containing sacculi treated with SDS are referred to below as native peptidoglycan-containing sacculi. For extraction of the secondary cell wall polymer, 10 mg of lyophilized native peptidoglycan-containing sacculi was suspended in 1 ml of formamide and incubated for 1 h at 100 or 150°C. As an alternative, the peptidoglycan-containing sacculi were extracted with 25 mM glycine-HCl buffer (pH 2.5) for 30 min at 100°C or with 0.1 M HCl for 30 min at 60°C. For cleaving possible phosphodiester linkages between the cell wall polymer and the peptidoglycan, peptidoglycan-containing sacculi were incubated with 40 or 48% hydrofluoric acid (HF) at 0 or 4°C (9) for 7 to 96 h (see Table 2). After centrifugation at 40,000 × *g* for 20 min at 4°C, the pellets were washed once with the respective extracting agent and three times with distilled water. The pellets were suspended in 1 ml of distilled water, frozen at −20°C, lyophilized, and used for chemical analyses and S-layer recrystallization experiments. The supernatant fluids obtained by extraction of the peptidoglycan-containing sacculi with 48% HF were used for isolation of the secondary cell wall polymer.

HF extraction of S-layer self-assembly products. For extraction of the secondary cell wall polymer associated with the S-layer self-assembly products, 10-mg portions of lyophilized samples were suspended in 1 ml of 40% HF and incubated for 18 h at 4°C. After the HF-extracted cell wall polymer was removed from the S-layer self-assembly products by centrifugation at 40,000 × *g* for 20 min at 4°C, the pellet was washed at least three times with distilled water. The HF-extracted S-layer self-assembly products were analyzed by SDS-PAGE, used for chemical analysis, and recrystallization experiments.

Recrystallization experiments. Native peptidoglycan-containing sacculi and those extracted under conditions listed in Table 2 were used for S-layer recrystallization experiments. For this purpose, 2.5 mg of lyophilized peptidoglycan-containing sacculi and 2.5 mg of lyophilized S-layer self-assembly products were suspended in 5 ml of 5 M GHCl in 50 mM Tris-HCl buffer (pH 7.2), stirred for 2 h at 20°C, and dialyzed against distilled water overnight at 4°C (30, 31). Subsequently, the samples were investigated by negative staining and ultrathin sectioning (19).

Isolation of the HF-extracted secondary cell wall polymer. After the cell wall polymer was extracted with 48% HF from native peptidoglycan-containing sacculi or with 40% HF from S-layer self-assembly products, the suspensions were centrifuged at 40,000 × *g* for 20 min at 4°C. The clear supernatants were carefully removed from the pellets and were used for precipitation of the secondary cell wall polymer. This was done by addition of 5 volumes of chilled ethanol (−20°C) absolute (5). After incubation for 18 h at −20°C, the precipitate was sedimented at 20,000 × *g* for 15 min at −10°C. The pellet was washed twice with chilled ethanol and was finally dissolved in distilled water. The clear solution was dialyzed (Biomol, membrane type 8; molecular weight cutoff, 12,000 to 16,000) against distilled water for 24 h at 4°C, frozen at −20°C, and lyophilized. For determining the molecular weight and investigating the homogeneity of the secondary cell wall polymer, gel permeation chromatography was performed. For this purpose, 10 mg of the cell wall polymer was dissolved in 1 ml of 150 mM NaCl and applied to a Sephadex G-150 column with a total volume of 181 ml. Calibration of the column was done with a Pharmacia LMW gel filtration calibration kit (Pharmacia, Uppsala, Sweden). Chemical analyses of the secondary cell wall polymer were performed before and after purification by gel permeation chromatography.

TABLE 1. Chemical analysis of native (SDS-extracted) peptidoglycan-containing sacculi from *B. stearothermophilus* PV72/p2

Constituent	Molar ratio[a]	
	Theoretical[b]	Actual[c]
MurNAc	1	1.00
GlcNAc	1	2.05 (1.05)
Ala	1.5	1.54
DAP	1	0.97
ManNAc		0.30 (0.30)

[a] Relative to that for Glu, which was assigned a value of 1.
[b] According to A1γ chemotype.
[c] Excess GlcNAc (value obtained by subtraction of the amount of MurNAc from the GlcNAc content) (given in parentheses) was attributed to the presence of a secondary cell wall polymer. The molar ratio between excess GlcNAc and ManNAc was 3.5:1.

Chemical analyses. Cell wall fragments, S-layer self-assembly products, and peptidoglycan-containing sacculi were used for amino acid, amino sugar, and neutral sugar analyses. For amino acid and amino sugar analyses, the samples were hydrolyzed with 6 N HCl for 6 h at 110°C. After modification with sodium borohydride and *o*-phthalaldehyde (1), amino acids and amino sugars were analyzed by high-pressure liquid chromatography. Neutral sugars were liberated by hydrolysis of lyophilized samples with 2.2 M trifluoroacetic acid for 4 h at 110°C. After drying with nitrogen, the samples were dissolved in distilled water and applied to a DIONEX DX-300 gradient chromatography system. The detailed procedure for neutral sugar analysis is given in references 24 and 29.

Chemical modification of native and HF-extracted peptidoglycan-containing sacculi. The numbers of free carboxylate groups in native and HF-extracted peptidoglycan-containing sacculi were determined according to the procedure of Weigert and Sára (34). After activation of free carboxylate groups with 1-ethyl-3,3(dimethylaminopropyl)carbodiimide and covalent binding of glycine methyl ester, the glycine contents of modified samples were determined by amino acid analysis. Free amino groups were modified by binding preactivated leucine (butyloxycarbonyl–L-leucine–*N*-hydroxysuccinimideester) under conditions specified in reference 34. The leucine content was determined by amino acid analysis.

Studies on the importance of the secondary cell wall polymer for the in vitro self-assembly process. The amounts of secondary cell wall polymer associated with S-layer self-assembly products formed at different periods during dialysis of the GHCl-extracted S-layer protein were investigated. For this purpose, samples were taken at defined intervals and centrifuged at 20,000 × *g* for 5 min at 4°C, and the percent assembled S-layer protein (degree of assembly [DA]) as well as the total sugar contents in both fractions were determined according to the methods described in references 32 and 33. When the DA was 20%, the clear supernatant containing nonassembled (monomeric or oligomeric) S-layer protein was used for recrystallization experiments. For this purpose, electron microscope (EM) grids precoated with poly-L-lysine (0.1% in distilled water) were placed on a drop of the S-layer protein solution and incubated under conditions specified in a previous paper (22). The S-layer protein solution was also injected into the subphase of a Langmuir-Blodgett trough on which a monolayer of dipalmitoylphosphatidylethanolamine (DPPE) was established. The experiments with the Langmuir-Blodgett trough were carried out as described in a previous paper (23). The ability of the S-layer monomers to recrystallize into a monolayer was checked by negative staining.

Investigation of the affinity between proteolytic cleavage fragments of the S-layer protein and native peptidoglycan-containing sacculi. Proteolytic cleavage of the S-layer protein with endoproteinase Glu-C (*Staphylococcus aureus* V8 protease) was done in SDS buffer (0.1% SDS in 50 mM Tris-HCl buffer, pH 7.8) for 4 h at 37°C (24). Lyophilized native peptidoglycan-containing sacculi (1 mg) were added to the solution of the proteolytically degraded S-layer protein, and the suspension was incubated for 1 h at 20°C. Subsequently, the suspension was centrifuged at 40,000 × *g* for 20 min at 20°C, and both the clear supernatant and the pellet were subjected to SDS-PAGE using a 10 or 6% separation gel (19). After SDS-PAGE, the protein bands were blotted to a polyvinylidene difluoride membrane (Immobilon PSQ; Millipore) and subjected to N-terminal sequencing as described in previous papers (3, 4).

RESULTS

Chemical analyses of native and extracted peptidoglycan-containing sacculi. The results of the amino acid and amino

TABLE 2. Results from extraction of the secondary cell wall polymer from native peptidoglycan-containing sacculi of *B. stearothermophilus* PV72/p2

Sample h (temp [°C])	Amt[a]					% Extracted[b]		Recrystallization[c]
	MurNAc	Dap	Ala	Excess GlcNAc[d]	ManNAc	GlcNAc[d]	ManNAc	
Native	1.00	0.97	1.54	1.05	0.30			+
Formamide, 1 h (100)	1.15	0.97	1.57	0.62	0.18	41	40	+
Formamide, 1 h (150)	1.15	0.94	1.47	0.02	0.09	98	70	−
0.1 M HCl, 0.5 h (60)	1.00	1.00	1.52	0.84	0.27	20	10	+
pH 2.5,[e] 0.5 h (100)	0.97	0.92	1.38	0.00	0.01	100	97	−
40% HF								
48 h (0)	1.02	1.10	1.50	0.79	0.24	25	20	+
66 h (4)	1.08	0.98	1.45	0.52	0.17	50	43	+
48% HF								
7 h (4)	1.13	1.14	1.63	0.56	0.22	47	27	+
18 h (4)	1.08	1.17	1.54	0.28	0.09	73	70	+/−
25 h (4)	1.13	1.18	1.68	0.07	0.06	93	80	−
48 h (4)	1.13	1.03	1.51	0.00	0.07	100	77	−
96 h (4)	0.96	0.94	1.46	0.00	0.00	100	100	−

[a] Relative to that of Glu, which was assigned a value of 1. Excess GlcNAc was calculated by subtraction of the amount of MurNAc from the total GlcNAc content. Together with ManNAc, excess GlcNAc was attributed to the secondary cell wall polymer.
[b] From the secondary cell wall polymer.
[c] Results from negative staining and ultrathin sectioning.
[d] Samples from which more than 90% of excess GlcNAc was extracted are underlined.
[e] 25 mM glycine-HCl buffer.

sugar analyses of native peptidoglycan-containing sacculi from *B. stearothermophilus* PV72/p2 are given in Table 1. With exception of GlcNAc, the molar ratios of all other peptidoglycan constitutents (Glu, Ala, diaminopimelic acid [DAP], and *N*-acetylmuramic acid [MurNAc]) corresponded to the directly cross-linked A1γ chemotype typical of *B. stearothermophilus* strains (26). In comparison to MurNAc, the GlcNAc content was twice as much as the theoretical value (molar ratio of GlcNAc to MurNAc, 2.05:1). Since the molar ratio of GlcNAc to MurNAc is 1 in different peptidoglycan chemotypes (26), the surplus of GlcNAc indicated the presence of a secondary cell wall polymer. In addition to GlcNAc, ManNAc was identified as the second amino sugar. The molar ratio of GlcNAc to ManNAc attributed to the secondary cell wall polymer was 3.5 to 1.

In Table 2, the results from chemical analyses and recrystallization experiments using native and differently extracted peptidoglycan-containing sacculi are summarized. For calculation of GlcNAc belonging to the secondary cell wall polymer (excess GlcNAc), the MurNAc content of each sample was subtracted from the total GlcNAc content. For calculating the percent extracted ManNAc and GlcNAc for each sample in Table 2, the amounts determined for native peptidoglycan-containing sacculi were assigned a value of 100%.

As shown in Table 2, about 20 to 50% of the secondary cell wall polymer could be extracted by treating native peptidoglycan-containing sacculi with formamide at 100°C for 1 h, 0.1 M HCl at 60°C for 30 min, 40% HF at 0 or 4°C, and 48% HF for 7 h at 4°C. A higher portion of the secondary cell wall polymer (up to 100% of excess GlcNAc and up to 80% of ManNAc) was removed by incubating native peptidoglycan-containing sacculi with formamide at 150°C for 1 h, 25 mM glycine-HCl buffer (pH 2.5) at 100°C for 30 min, or 48% HF for 18, 25, and 48 h at 4°C (Table 2). Complete extraction of the secondary cell wall polymer was achieved when the incubation time with 48% HF was extended to 96 h.

In comparison to native peptidoglycan-containing sacculi the molar ratios between MurNAc, DAP, Ala, and Glu were not significantly changed in the extracted samples, indicating that neither chemical hydrolysis nor enzymatic degradation of the peptidoglycan had occurred (Table 2). This was also confirmed by negative staining and ultrathin sectioning in which extracted peptidoglycan-containing sacculi revealed a shape typical of native samples (not shown).

Negative staining and ultrathin sectioning confirmed that the GHCl-extracted S-layer protein was able to recrystallize on native peptidoglycan-containing sacculi into the oblique S-layer lattice typically formed by the SbsB (not shown). Complete recrystallization of the S-layer protein was also observed when at least 50% of the secondary cell wall polymer remained in the extracted peptidoglycan-containing sacculi (Fig. 1a; formamide at 100°C, 0.1 M HCl at 60°C, 40% HF, and 48% HF for 7 h). In ultrathin-sectioned preparations, a three-layered cell envelope profile was detected, confirming that after recrystallization of the S-layer protein, complete outer and inner S-layers were formed (Fig. 1a). On peptidoglycan-containing sacculi extracted with formamide at 150°C, 25 mM glycine-HCl buffer (pH 2.5), and 48% HF for 25, 48, and 96 h, the S-layer protein was not able to bind and recrystallize (not shown). According to chemical analyses, these samples represented almost pure peptidoglycan (Table 2). Small patches of an outer and an inner S-layer could be detected on peptidoglycan-containing sacculi treated with 48% HF for 18 h (Fig. 1b). Under these conditions, approximately 70% of the secondary cell wall polymer had been extracted (Table 2).

Analyses of native and HF-extracted S-layer self-assembly products. Results from amino acid and amino sugar analyses of the GHCl-extracted, dialyzed, and recrystallized S-layer pro-

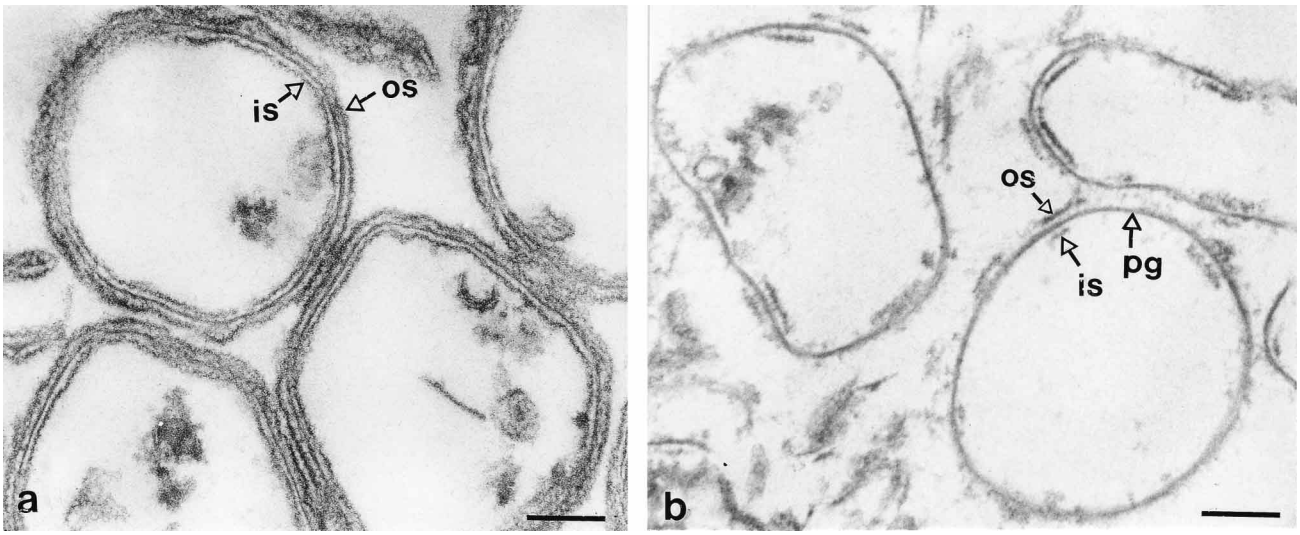

FIG. 1. Ultrathin-sectioned preparations of differently extracted peptidoglycan-containing sacculi (pg) from *B. stearothermophilus* PV72/p2 after recrystallization of the S-layer protein. (a) Complete outer (os) and inner (is) S-layers; (b) only small patches of outer and inner S-layers visible. Bars, 200 nm.

tein before and after extraction with 40% HF are summarized in Table 3. Before treatment with 40% HF, both GlcNAc and ManNAc could be detected in S-layer self-assembly products. The molar ratio of GlcNAc to ManNAc was 4.1:1 and therefore in good accordance with the results obtained for the secondary cell wall polymer in native peptidoglycan-containing sacculi. Even after extraction of the secondary cell wall polymer with 40% HF, the S-layer protein migrated as a single band on SDS gels (not shown). The apparent molecular weight of the HF-extracted S-layer protein was identical to that of native S-layer subunits, which confirmed that the S-layer protein was not degraded during the extraction procedure. After disintegration of HF-extracted S-layer self-assembly products with 5 M GHCl, the S-layer subunits still recognized the native peptidoglycan-containing sacculi as binding site but had lost the ability to recrystallize into the oblique lattice. In negatively stained preparations, only a granular structure was visible (not shown).

TABLE 3. Chemical analysis of native S-layer self-assembly products and S-layer self-assembly products extracted with 40% HF for 18 h at 4°C

Amino acid or amino sugar	S-layer protein (mol%)	
	Native	HF-extracted
Asx	14.7	15.5
Glx	8.8	8.6
Ser	6.3	6.7
His	0.5	0.3
Gly	8.1	7.5
Thr	9.4	9.9
Arg	2.2	2.1
Ala	9.6	9.9
Tyr	2.2	2.0
Val	11.3	12.0
Ile	4.4	4.4
Phe	3.9	3.9
Leu	6.3	6.6
Lys	8.7	9.2
GlcNAc[a]	3.3	
ManNAc[a]	0.8	

[a] The molar ratio of GlcNAc to ManNAc was 4.1:1.

Characterization of the HF-extracted cell wall polymer. Amino acid and amino sugar analyses of the cell wall polymer extracted from native peptidoglycan-containing sacculi with 48% HF for 7 to 96 h at 4°C revealed that the molar ratio of GlcNAc to ManNAc was about 4:1 (Table 4). The glucose content was <1%. Since only negligible amounts of Glu, Ala, and DAP were detected in the HF-extracted cell wall polymer, it was confirmed that it is composed mainly of GlcNAc and ManNAc and that the peptidoglycan was not degraded during the extraction procedure. The cell wall polymer isolated from S-layer self-assembly products revealed a similar molar ratio of GlcNAc to ManNAc (4.1:1). As determined by gel permeation chromatography, the cell wall polymer extracted from both materials was eluted as a single peak with an estimated molecular weight of 24,000. The cell wall polymer represented approximately 20% of the peptidoglycan-containing-sacculus dry weight and about 3% from the S-layer self-assembly products. The glucose content of the cell wall polymer purified by gel permeation chromatography was 0.4%, and the molar ratio of GlcNAc to ManNAc was 4:1.

Chemical modification of native and HF-extracted peptidoglycan-containing sacculi. Amino acid analysis of 1-ethyl-3,3

TABLE 4. Chemical analyses of the secondary cell wall polymer extracted from native peptidoglycan-containing sacculi from *B. stearothermophilus* PV72/p2 with 48% HF at 4°C for 7 to 96 h

Extraction (h)	Amt[a]			
	GlcNAc	Glu	Ala	Dap
7	3.9	<0.1	0	0
18	4.1	<0.1	0	0
25	4.0	<0.1	0	0
48	4.8	<0.05	<0.05	<0.1
96	4.2	<0.05	<0.05	<0.1

[a] Relative to that of ManNAc, which was assigned a value of 1. The MurNAc contents of all samples were 0.

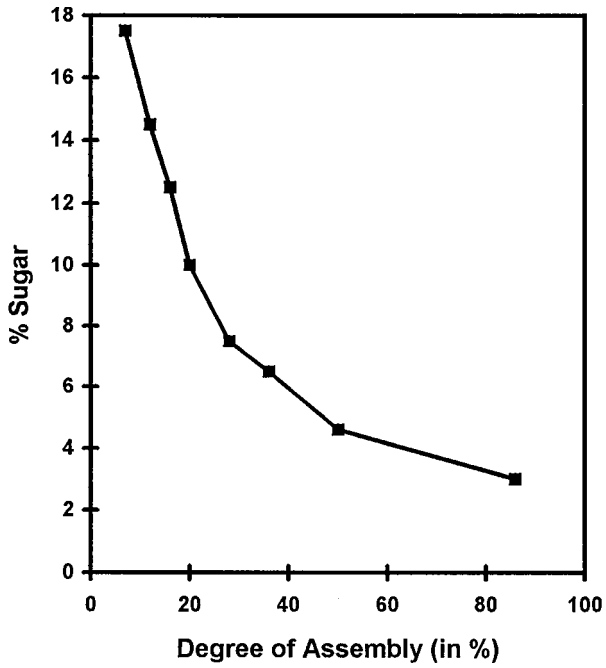

FIG. 2. Correlation between the sugar content of S-layer self-assembly products from *B. stearothermophilus* PV72/p2 and the DA. The sugar content is given as percentage of the S-layer protein.

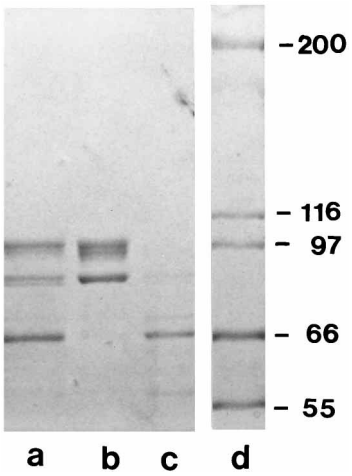

FIG. 4. SDS-PAGE patterns demonstrating the affinity of S-layer protein cleavage fragments for binding to native peptidoglycan-containing sacculi from *B. stearothermophilus* PV72/p2. Lanes: a, S-layer protein degraded with endoproteinase Glu-C; b, S-layer protein and cleavage fragments which had bound to the native peptidoglycan-containing sacculi; c, S-layer protein cleavage fragment detected in the clear supernatant; d, molecular weight standard. Molecular weights are given in thousands.

(dimethylaminopropyl)carbodiimide-activated, glycine methyl ester-modified native, and HF-extracted peptidoglycan-containing sacculi revealed that the molar ratio of DAP to Gly was 1:2. Thus, in both types of samples two carboxylate groups were available per DAP residue. Neither native nor HF-extracted peptidoglycan-containing sacculi were capable of binding preactivated Leu, which indicated the absence of accessible free amino groups. Since native and HF-extracted samples gave identical results, the secondary cell wall polymer does not contribute significant free amino and carboxylate groups to the peptidoglycan-containing sacculi.

Self-assembly and recrystallization experiments. In Fig. 2, the correlation between the DA and the sugar content of S-layer self-assembly products formed during dialysis of the GHCl-extracted S-layer protein is shown. In general, the sugar content of S-layer self-assembly products decreased with increasing DA. For example, a total sugar content of 17.5% was determined for S-layer self-assembly products sedimented at the beginning of the dialysis procedure at a DA of 7%, while the sugar content of S-layer self-assembly products analyzed at the end of the dialysis procedure at a DA of 86% had de-

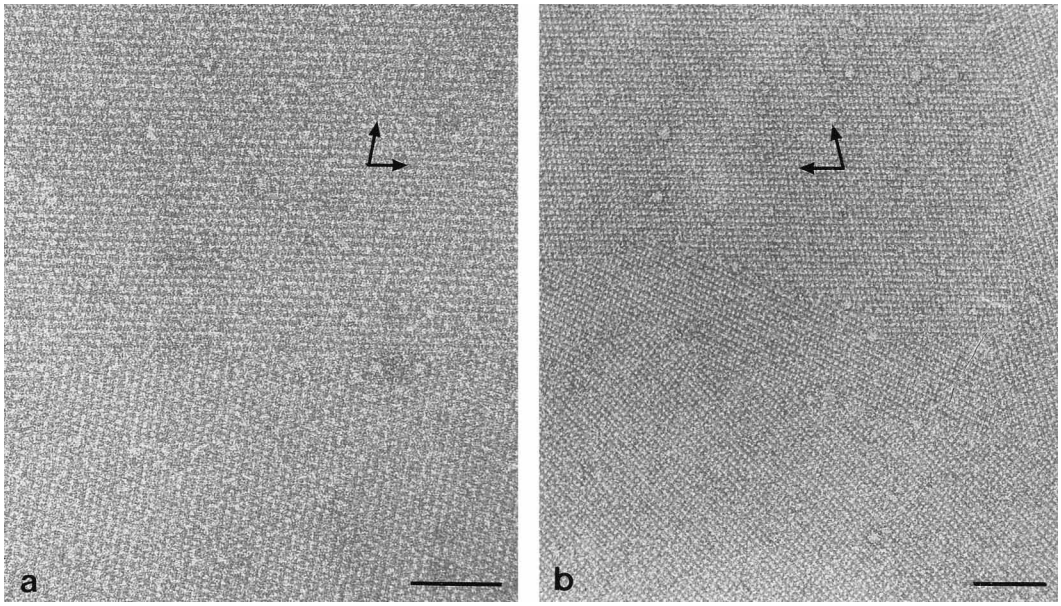

FIG. 3. Negative staining of the S-layer protein from *B. stearothermophilus* PV72/p2 recrystallized into a monolayer on a poly-L-lysine-coated EM grid (a) or on a DPPE monolayer (b). Arrows, base vectors. Bars, 100 nm.

creased to 3%. This value corresponded exactly to the amount of secondary cell wall polymer associated with S-layer self-assembly products used for chemical analysis and HF extraction experiments.

S-layer self-assembly products sedimented at a DA of 20% had a total sugar content of 10% (Fig. 2). At the same time, the sugar content of the nonassembled monomeric or oligomeric S-layer protein present in the clear supernatant was in the range of 1%. This nonassembled S-layer protein could perfectly recrystallize into closed monolayers on poly-L-lysine-coated EM grids or on a DPPE monolayer generated by the Langmuir-Blodgett technique (Fig. 3). As derived from the orientation of the base vectors of the oblique S-layer lattice in negatively stained preparations, the subunits had bound with their outer face to the poly-L-lysine-coated EM grids and with their inner face to the DPPE monolayer (Fig. 3).

Proteolytic degradation of the S-layer protein with endoproteinase Glu-C. After proteolytic degradation of the S-layer protein with endoproteinase Glu-C, two protein bands with apparent molecular weights of 66,000 and 85,000 and a more diffuse protein band with an apparent molecular weight of ≤97,000 were observed on SDS gels (Fig. 4, lane a). The presence of a protein band with an apparent molecular weight of 97,000 indicated that not the whole amount of S-layer protein was degraded by the protease.

For investigating the affinity between the different S-layer protein cleavage fragments and native peptidoglycan-containing sacculi, the latter were suspended in a solution of the proteolytically degraded S-layer protein. After incubation and sedimentation, the pellet consisting of peptidoglycan-containing sacculi with the bound S-layer protein cleavage fragments and the clear supernatant were investigated by SDS-PAGE. As shown in Fig. 4, lane b, only protein bands with apparent molecular weights of ≤97,000 and 85,000 showed affinity for binding to native peptidoglycan-containing sacculi. N-terminal sequencing revealed that these protein bands had an N-terminal region identical to that of the whole S-layer protein (A-S-F-T-D-V-A-P-Q-Y-K-D). In contrast to the results with the higher-molecular-weight protein bands, the S-layer protein cleavage fragment with an apparent molecular weight of 66,000 was detected only in the clear supernatant (Fig. 4, lane c). The N-terminal sequence of this cleavage fragment showing no binding to native peptidoglycan-containing sacculi was L-T-S-S-N-T-N-I-V-V. This sequence began at a Leu (L) in position 300 of the mature S-layer protein (10). Thus, proteolysis of the S-layer protein with endoproteinase Glu-C had occurred between Glu (E) in position 299 and Leu in position 300.

DISCUSSION

The first indication that secondary cell wall polymers could be responsible for binding of S-layer proteins to the peptidoglycan-containing layer was reported by Hastie and Brinton (7) for *B. sphaericus*. Later, a similar observation was made for *Lactobacillus buchneri* (16): extraction, chemical modification, and recrystallization experiments confirmed that a neutral sugar polymer, and not the peptidoglycan or the teichoic acids, functions as a binding site for the S-layer protein (16). More recently it was shown that a secondary cell wall polymer composed of GlcNAc and Glc anchors the S-layer protein from *B. stearothermophilus* PV72/p6 to the rigid cell wall layer (25).

In the present study we could demonstrate that a secondary cell wall polymer composed of GlcNAc and ManNAc in a molar ratio of 4:1 is responsible for binding of the S-layer protein from *B. stearothermophilus* PV72/p2 to the peptidoglycan-containing layer. Preliminary NMR studies showed that both amino sugars are acetylated (9a). The estimated molecular weight of the cell wall polymer was 24,000, indicating that a single polymer chain consisted of approximately 120 monosaccharide residues. Incubation of proteolytic cleavage fragments of the S-layer protein with native peptidoglycan-containing sacculi further revealed that the N-terminal part possessing a typical SLH domain (10) had affinity to the secondary cell wall polymer. A C-terminal cleavage fragment carrying two-thirds of the amino acids of the whole S-layer protein did not recognize the native peptidoglycan-containing sacculi. On the other hand, the whole S-layer protein could not bind to HF-extracted peptidoglycan-containing sacculi which according to chemical analyses represented pure peptidoglycan.

The high affinity between the secondary cell wall polymer and the S-layer protein from *B. stearothermophilus* PV72/p2 was also demonstrated by the presence of the cell wall polymer in the fraction of the S-layer self-assembly products. In the course of cell growth and cell wall autolysis, both peptidoglycan strands and cell wall polymer chains are released from the rigid cell wall layer, but only the secondary cell wall polymer remained attached to the S-layer protein. The cell wall polymer associated with the S-layer protein had a molecular weight and composition identical to those of the polymer detected in native peptidoglycan-containing sacculi and was extracted from cell wall fragments together with the S-layer protein with 5 M GHCl. During dialysis and refolding of the GHCl-extracted S-layer protein, the cell wall polymer could reassociate with the respective N-terminal S-layer protein domains and was accumulated in the fraction of the S-layer self-assembly products. Moreover, the cell wall polymer could not be removed by washing the self-assembly products with various buffers or salt solutions (data not shown).

Studies on the kinetics of the in vitro self-assembly of the GHCl-extracted S-layer protein revealed that S-layer self-assembly products formed at the beginning of the dialysis procedure had the highest amount of cell wall polymer associated. On the other hand, the sugar content of the soluble nonassembled S-layer protein was generally very low (1 to 2%). For example, S-layer self-assembly products analyzed at a DA of 20% had a sugar content of 10%, while the sugar content of the nonassembled S-layer protein was only 1%. From the amount of cell wall polymer associated with the S-layer self-assembly products and the recrystallization experiments, we propose that the secondary cell wall polymer could play an important role for the correct folding of the S-layer subunits and for initiating the in vitro self-assembly process. According to this hypothesis, the soluble S-layer protein showing a low sugar content could recrystallize into monolayers if appropriate surfaces such as poly-L-lysine-coated EM grids or Langmuir-Blodgett lipid films were available. In a previous study it was demonstrated that oligomeric precursors formed at the beginning of the in vitro self-assembly process represent the nucleation sites for S-layer lattice growth and for the formation of S-layer self-assembly products (8). This would explain why after initiation of the in vitro self-assembly, low concentrations of the secondary cell wall polymer in the fraction of the soluble S-layer protein were not disadvantageous for further S-layer lattice growth. The ability of the recombinant SbsB to assemble in the cytoplasm of *Escherichia coli* (10) confirmed that the secondary cell wall polymer is not necessary for the in vitro self-assembly of the GHCl-extracted S-layer subunits.

Cell wall fragments used in the present study had an S-layer protein content of 50%, which corresponds to complete outer and inner S-layers (21, 29). From the presence of an inner S-layer it can be concluded that an S-layer protein pool sufficient for the complete coverage of the cell surface was accu-

mulated in the meshwork of the rigid cell wall layer of whole cells grown in continuous culture under steady-state conditions (2). In this context, the question of the role of the secondary cell wall polymer for the formation and maintenance of the S-layer protein pool has to be asked. On the one hand, the cell wall polymer is able to interact with the N-terminal part of the S-layer protein and can keep the subunits stored within the peptidoglycan-containing layer. On the other hand, both the polymer chains as well as the peptidoglycan strands could function as spacers, thereby preventing the assembly of the S-layer subunits into macromolecular arrays during their export to the cell surface (2, 21). As derived from chemical analyses, the cell wall fragments consisted of 50% S-layer protein, 40% peptidoglycan, and 10% cell wall polymer. Since the estimated molecular weight of the cell wall polymer is 24,000 and that of the S-layer subunits is 97,000, on average 1.3 polymer chains or about 150 monosaccharide residues were available per S-layer subunit.

From our studies with *B. stearothermophilus* PV72/p2 it can be concluded that the N-terminal part of the S-layer protein recognizes a secondary cell wall polymer incorporated into the peptidoglycan. According to the proposed affinity between the secondary cell wall polymer and the N-terminal part of the S-layer protein, the S-layer subunits from *B. stearothermophilus* PV72/p2 did not bind and recrystallize on pure peptidoglycan and on peptidoglycan-containing sacculi from the wild-type strain which had an identical peptidoglycan chemotype but a different composition of the secondary cell wall polymer (25). Studies concerning the determination of the specific polymer binding site on the S-layer protein are in progress.

ACKNOWLEDGMENTS

This work was supported by the Fonds zur Förderung der Wissenschaftlichen Forschung of Austria, project S72/02, and by the Bundesministerium für Wissenschaft, Verkehr und Kunst.

We thank Harald F. Mayer for continuous cultivations, Thomas Dalik for amino acid analyses, and Sonja Zayni for neutral sugar analyses.

REFERENCES

1. **Altmann, F.** 1992. Determination of amino sugaras and amino acids in glycoconjugates using precolumn derivatization with o-phthalaldehyde. Anal. Biochem. **204:**215–219.
2. **Breitwieser, A., K. Gruber, and U. B. Sleytr.** 1992. Evidence for an S-layer protein pool in the peptidoglycan of *Bacillus stearothermophilus*. J. Bacteriol. **174:**8008–8015.
3. **Egelseer, E. M., I. Schocher, M. Sára, and U. B. Sleytr.** 1995. The S-layer from *Bacillus stearothermophilus* DSM 2358 functions as an adhesion site for a high-molecular-weight amylase. J. Bacteriol. **177:**1444–1451.
4. **Egelseer, E. M., I. Schocher, U. B. Sleytr, and M. Sára.** 1996. Evidence that an N-terminal S-layer protein fragment triggers the release of a cell-associated high-molecular-weight amylase in *Bacillus stearothermophilus* ATCC 12980. J. Bacteriol. **178:**5602–5609.
5. **Ekwunife, F. S., J. Singh, J., K. G. Taylor, and R. J. Doyle.** 1991. Isolation and purification of a cell wall polysaccharide of *Bacillus anthracis* (Sterne). FEMS Microbiol. Lett. **82:**257–262.
6. **Etienne-Toumelin, I., J.-C. Sirard, E. Duflot, M. Mock, and A. Fouet.** 1995. Characterization of the *Bacillus anthracis* S-layer: cloning and sequencing of the structural gene. J. Bacteriol. **177:**614–620.
7. **Hastie, A. T., and C. C. Brinton, Jr.** 1979. Specific interaction of the tetragonally arrayed protein layer of *Bacillus sphaericus* with its peptidoglycan sacculus. J. Bacteriol. **138:**1010–1021.
8. **Jaenicke, R., R. Welsch, M. Sára, and U. B. Sleytr.** 1985. Stability and self-assembly of the S-layer protein of the cell wall of *Bacillus stearothermophilus*. Biol. Chem. Hoppe-Seyler **366:**663–670.
9. **Jürgens, U. J., and J. Weckesser.** 1986. Polysaccharide covalently linked to the peptidoglycan of the cyanobacterium *Synechocystis* sp. strain PCC6714. J. Bacteriol. **168:**568–573.

9a. **Kosma, P.** Personal communication.

10. **Kuen, B., A. Koch, E. Asenbauer, M. Sára, and W. Lubitz.** 1997. Molecular characterization of the second S-layer gene *sbsB* of *Bacillus stearothermophilus* PV72 expressed by oxidative stress. J. Bacteriol. **179:**1664–1670.
11. **Kuen, B., M. Sára, and W. Lubitz.** 1996. Heterologous expression and self-assembly of the S-layer protein SbsA of *Bacillus stearothermophilus* in *Escherichia coli*. Mol. Microbiol. **19:**495–503.
12. **Kuen, B., U. B. Sleytr, and W. Lubitz.** 1994. Sequence analysis of the *sbsA* gene encoding the 130 kDa surface layer protein of *Bacillus stearothermophilus* PV72. Gene **145:**115–120.
13. **Lemaire, M., H. Ohayon, P. Gounon, T. Fujino, and P. Beguin.** 1995. OlpB, a new outer layer protein of *Clostridium thermocellum*, and binding of its S-layer-like domains to components of the cell envelope. J. Bacteriol. **177:** 2451–2459.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
15. **Lupas, A., H. Engelhardt, J. Peters, U. Santarius, S. Volker, and W. Baumeister.** 1994. Domain structure of the *Acetogenium kivui* surface layer revealed by electron crystallography and sequence analysis. J. Bacteriol. **176:**1224–1233.
16. **Masuda, K., and T. Kawata.** 1985. Reassembly of a regularly arrayed protein in the cell wall of *Lactobacillus buchneri* and its reattachment to cell walls: chemical modification studies. Microbiol. Immunol. **29:**927–938.
17. **Matuschek, M., G. Burchhardt, K. Sahm, and H. Bahl.** 1994. Pullulanase of *Thermoanaerobacterium thermosulfurigenes* EM1 (*Clostridium thermosulfurogenes*): molecular analysis of the gene, composite structure of the enzyme, and a common model for its attachment to the cell surface. J. Bacteriol. **176:**3295–3302.
18. **Matuschek, M., K. Sahm, A. Zibat, and H. Bahl.** 1996. Characterization of genes from *Thermoanaerobacterium thermosulfurigenes* EM1 that encode two glycosyl hydrolases with conserved S-layer-like domains. Mol. Gen. Genet. **252:**493–496.
19. **Messner, P., F. Hollaus, and U. B. Sleytr.** 1984. Paracrystalline cell wall surface layers of different *Bacillus stearothermophilus* strains. Int. J. Syst. Bacteriol. **34:**202–210.
20. **Olabarría, G., J. L. Carrascosa, M. A. de Pedro, and J. Berenguer.** 1996. A conserved motif in S-layer proteins is involved in peptidoglycan binding in *Thermus thermophilus*. J. Bacteriol. **178:**4765–4722.
21. **Pink, T., K. Langer, C. Hotzy, and M. Sára.** 1996. Regulation of S-layer protein synthesis of *Bacillus stearothermophilus* PV72 through variation of continuous cultivation conditions. J. Biotechnol. **50:**189–200.
22. **Pum, D., M. Sára, and U. B. Sleytr.** 1989. Structure, surface charge, and self-assembly of the S-layer lattice from *Bacillus coagulans* E38-66. J. Bacteriol. **171:**5296–5303.
23. **Pum, D., M. Weinhandl, C. Hödl, and U. B. Sleytr.** 1993. Large-scale recrystallization of the S-layer of *Bacillus coagulans* E38-66 at the air/water interface and on lipid films. J. Bacteriol. **175:**2762–2766.
24. **Sára, M., D. Pum, S. Küpcü, P. Messner, and U. B. Sleytr.** 1994. Isolation of two physiologically induced variant strains of *Bacillus stearothermophilus* NRS 2004/3a and characterization of their S-layer lattices. J. Bacteriol. **176:**848–860.
25. **Sára, M., B. Kuen, H. Mayer, F. Mandl, K. C. Schuster, and U. B. Sleytr.** 1996. Dynamics in oxygen-induced changes in S-layer protein synthesis from *Bacillus stearothermophilus* PV72 and the S-layer-deficient variant T5 in continuous culture and studies of the cell wall composition. J. Bacteriol. **178:** 2108–2117.
26. **Schleifer, K. H., and O. Kandler.** 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. **36:**407–477.
27. **Scholz, H., B. Kuen, W. Lubitz, and M. Sára.** S-layer variation in *Bacillus stearothermophilus* PV72. FEMS Microbiol. Rev., in press.
28. **Schuster, K. C., T. Pink, H. F. Mayer, and M. Sára.** Oxygen-triggered synchronized variant formation of the S-layer carrying *Bacillus stearothermophilus* PV72 during continuous cultivation. J. Biotechnol., in press.
29. **Schuster, K. C., H. F. Mayer, R. Kieweg, W. Hampel, and M. Sára.** 1995. A synthetic medium for continuous culture of the S-layer carrying *Bacillus stearothermophilus* PV72 and studies on the influence of growth conditions on cell wall properties. Biotechnol. Bioeng. **48:**66–77.
30. **Sleytr, U. B.** 1975. Heterologous reattachment of regular arrays of glycoprotein on bacterial surfaces. Nature **257:**400–402.
31. **Sleytr, U. B.** 1976. Self-assembly of the hexagonally and tetragonally arranged subunits of bacterial surface layers and their reattachment to cell walls. J. Ultrastruct. Res. **55:**360–377.
32. **Sleytr, U. B., M. Sára, Z. Küpcü, and P. Messner.** 1986. Structural and chemical characterization of S-layers of selected strains of *Bacillus stearothermophilus* and *Desulfotomaculum nigrificans*. Arch. Microbiol. **146:**19–24.
33. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goerke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150:**76–85.
34. **Weigert, S., and M. Sára.** 1996. Ultrafiltration membranes prepared from bacterial cell surface layers as model systems for studying the influence of surface properties on protein adsorption. J. Membr. Sci. **121:**185–196.